Master's Thesis

Decolorization of melanin by lignin peroxidase with *in-situ* generated H_2O_2 for whitening cosmetics application

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2020

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A thesis/dissertation
Submitted to the Graduate School of UNIST
In partial fulfillment of the
Requirements for the degree of
Master of Science

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12. 02. 2019
Approved by

Advisor
Yong Hwan Kim

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12. 02. 2019

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Abstract

Lignin peroxidase is promising ingredient for skin whitening cosmetics because it is capable of oxidizing melanin with high redox potential. Crude lignin peroxidase from fungal fermentation is usually utilized owing to its difficulties of expression and purification. Lignin peroxidase is hard to express and purify so it was usually applied as crude form for cosmetics. In this study, lignin peroxidase isozyme H8 (LiPH8) from *Phanerochaete chrysosperium* was expressed in E. coli and purified for further use of melanin decolorization. The pH optimum for the decolorization of melanin was pH 4.0. Melanin decolorization efficiency was reached to 73% by intermittent addition of hydrogen peroxide (H_2O_2), since excessive concentration of H_2O_2 converts LiPH8 into compound III which is the inactivated form of lignin peroxidase. Considering that the intermittent supply of H_2O_2 is not practical for cosmetic applications, glucose oxidase from *aspergillus niger* (GOx) was utilized for *in-situ* generation of H_2O_2. GOx and β-D-glucose concentration was optimized and melanin decolorization efficiency reached 63.3% within 1h. LiPH8 retained its activity for 8h with 84.0% of melanin decolorization efficiency. With these results, melanin decolorization catalyzed by LiPH8 with H_2O_2 generated from GOx was proven to be a prospective approach for skin whitening applications.

Contents

List of figures and tables

Nomenclature

LiPH8: Lignin peroxidase isozyme H8 from *Phanerochaete chrysosporium*

GOx: Glucose oxidase from *Aspergillus niger*

Glucose: β-D-glucose

VA: 3,4-Dimethylbenzyl alcohol, veratryl alcohol

H_2O_2: Hydrogen peroxide

PTCA: Pyrrole-2,3,5-tricarboxylic acid

L-DOPA: L-3,4-dihydroxyphenylalanine

TRP: Tyrosinase related protein

***E. coli*:** *Escherichia coli*

BR buffer: Britton-Robinson buffer

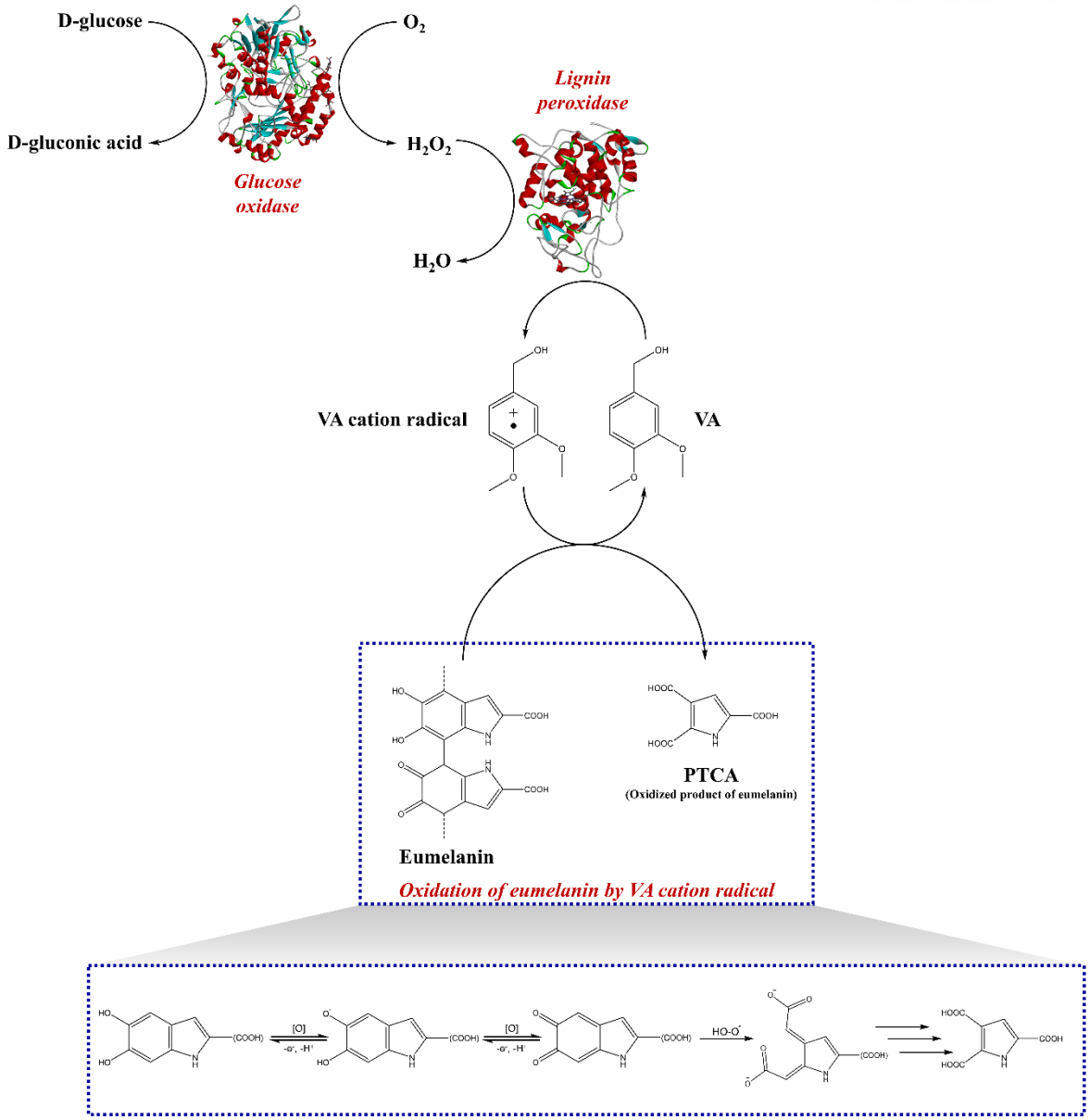
1. Introduction

Melanin is pigmented polymer that mainly determines the color of the skin. Melanin is produced by specialized epidermal cell called melanocyte and multiple genes regulates it [1]. Melanin also protects the skin from UV radiation by absorbing or refract it away from skin tissues [2]. Humans have two types of melanin: pheomelanin and eumelanin. Eumelanin have a brown or black color but pheomelanin shows a red or yellow color [3]. Asians have more darker color since relatively high concentration of eumelanin on their skin [4].

Therefore, especially for Asians, demand for skin whitening is increasing and researches on decolorization of melanin are ongoing actively. Generally, in whitening cosmetics, the active ingredients which prevent the production of melanin in the skin are obtained from natural sources. These ingredients inhibit melanin production through tyrosinase inhibition, pigment synthesis inhibition and other mechanisms [5]. Among them, most researchers focused on tyrosinase inhibitors to reduce production of melanin [6,7]. For example, rhododenol hinders the reaction which converts L-DOPA (L-3,4-dihydroxyphenylalanine) to DOPAquinone and blocks melanin biosynthetic pathway as a result. Azelaic acid [9], kojic acid [8], aloesin and arbutin [10] are also well-known chemicals as tyrosinase inhibitor which are employed for cosmetics. Besides tyrosinase inhibitors, there are other alternative mechanisms such as antioxidants which prevent oxidative damage of skin, tyrosinase related protein inhibitors, melanosome transfer inhibitors. Butin obtained from *Spatholobus suberectus* effectively inhibits tyrosinase related proteins and frequently used as whitening agent [11]. However, these chemical inhibitors of melanin synthesis were found to have potential side effects like vitiligo [12]. Accordingly, the whitening efficacy was evaluated by Tain et al. (2009) for arbutin containing cosmetic products on a human skin pigmentation spot models and concluded that the 3% arbutin has whitening effect to certain level but arbutin can result in cytotoxicity in higher concentration [13]. Therefore, the use of melanogenesis inhibitor for whitening cosmetics should be refrained. For this reason, studies of enzyme-catalyzed melanin decolorization are underway since enzymes have great advantage of high selectivity and less toxicity compare to chemicals. Lignin peroxidase, manganese peroxidase, dye-decolorizing peroxidase and laccase are previously tested for decolorization of melanin [14-18]. Lignin peroxidase is the most promising enzyme among them to decolorize melanin efficiently sine it has high redox potential which can oxidize veratryl alcohol (VA). Therefore, lignin peroxidase is expected to oxidize the melanin easily than other enzymes which have lower redox potential for melanin oxidation [19]. However, in spite of its high potential as skin whitening agent, lignin peroxidase was studied only as crude form due owing its difficult expression and complicated purification since 17 isozymes which have similar properties are included in the original host, *Phanerochaete chrysosporium* [20]. Moreover,

lignin peroxidase is vulnerable to inactivation in high concentration of hydrogen peroxide (H_2O_2) and melanin decolorization is also result of oxidation of melanin which consumes H_2O_2 as fuel [21]. So, inactivation of lignin peroxidase by H_2O_2 is a main obstacle to get high melanin decolorization efficiency.

In present work, to get the enzyme with high purity, lignin peroxidase isozyme H8 (LiPH8) was expressed in *Escherichia coli*. Recombinant LiPH8 was further utilized for melanin decolorization, instead of crude enzyme from the culture media of white-rot fungi as *Woo et al.* [18]. Furthermore, H_2O_2 generation system by glucose oxidase (GOx) and glucose was incorporated with LiPH8 for decolorization of melanin to prevent inactivation of lignin peroxidase by high concentration of H_2O_2. Since GOx can generate H_2O_2 continuously with low concentration from molecular oxygen and β-D-glucose (glucose), significant increase of melanin decolorization efficiency was expected. Thus, this approach can provide a foundation for skin whitening applications.



Scheme 1. Outline of melanin decolorization catalyzed by lignin peroxidase and glucose oxidase. The redox mediator, the veratryl alcohol cation radical decolorizes eumelanin and change it into PTCA (pyrrole-2,3,5-tricarboxylic acid) which is possible candidate of oxidize form of melanin.

2. Results and discussion

To satisfy the increasing demand for active reagents for the skin whitening cosmetics, the cosmetic companies have started to incorporate the chemicals such as tyrosinase inhibitors [22], glutathione [23], hydroquinone [24, 25], topical corticosteroids [26], topical retinoids [27], cysteine [28], and other reagents [29, 30]. These chemical active reagents can be obtained by synthesis or extraction from natural organisms and these substances disrupt the melanin biosynthesis in melanocytes epidermis [5]. However, the greater part of these reagents has revealed to be cytotoxic. So, it is questionable for their use as cosmetic ingredients. Therefore, the present study utilized enzyme to replace harmful chemical-based ingredients as active reagent for skin whitening cosmetics. Additionally, enzymes have advantages like high selectivity and specificity with no toxicity [31]. Therefore, this research strives to apply the recombinant LiPH8 for melanin decolorization as non-toxic active ingredient in whitening cosmetic formulations.

Initially, the optimum pH and ionic strength was determined then H_2O_2 effect on decolorization of melanin was analyzed. The effect of H_2O_2 should be determined, since the high concentration of it can inactivate lignin peroxidase [21]. Accordingly, continuous supply of low-concentration H_2O_2 was required to get the highest melanin decolorization efficiency. In order to achieve this goal, GOx was utilized for H_2O_2 generation with melanin decolorization catalyzed by LiPH8.

2.1 Melanin decolorization efficiency with different pH and ionic strength conditions

VA cation radical ($VA^{\cdot+}$) is a redox mediator for melanin decolorization which is produced from VA by lignin peroxidase (Scheme 1). Since VA is oriented from white-rot fungi as natural compound, it has the advantage of less toxicity than synthetic mediators [32]. Melanin decolorization study with crude laccases were reported previously [14]. They showed that the laccase decolorized melanin well in pH 4.0 to pH 6.5 and laccase had optimum pH in this range. Similarly, in this experiment, the effect of varying pH from 3.0 to 6.0 on 1 h melanin decolorization was analyzed. In case of LiPH8, specific activity for VA was highest at pH 3.0. However, melanin was precipitated at pH 3.0 and could not observe melanin decolorization (Fig. 1). Synthetic melanin has carboxyl group which has advantage for solubility in high pH condition (Scheme 1). Addition of LiPH8 accelerated the melanin precipitation due to aggregation of melanin and the enzyme. Melanin precipitation also occurred at ionic strength of 500 mM and 1000 mM while melanin decolorization was observed in lower ionic strengths 0 mM and

100 mM (Table 1). Therefore, the subsequent experiments were conducted in BR buffer with 0.1 M of ionic strength. Melanin and redox mediator should directly interact or the melanin decolorization will be reduced since the VA cation radical has a very short lifetime [33, 34]. The melanin decolorization efficiency was significantly decreased at pH 5.0 and pH 6.0 while it reached over 30% at pH 4.0 in 1 h since the optimum pH of LiPH8 for VA oxidation was around pH 3.0. It is clear that high specific activity of LiPH8 for VA is essential for higher decolorization efficiency (Fig. 1). Therefore, all the melanin decolorization were carried out in pH 4.0 for the following reactions.

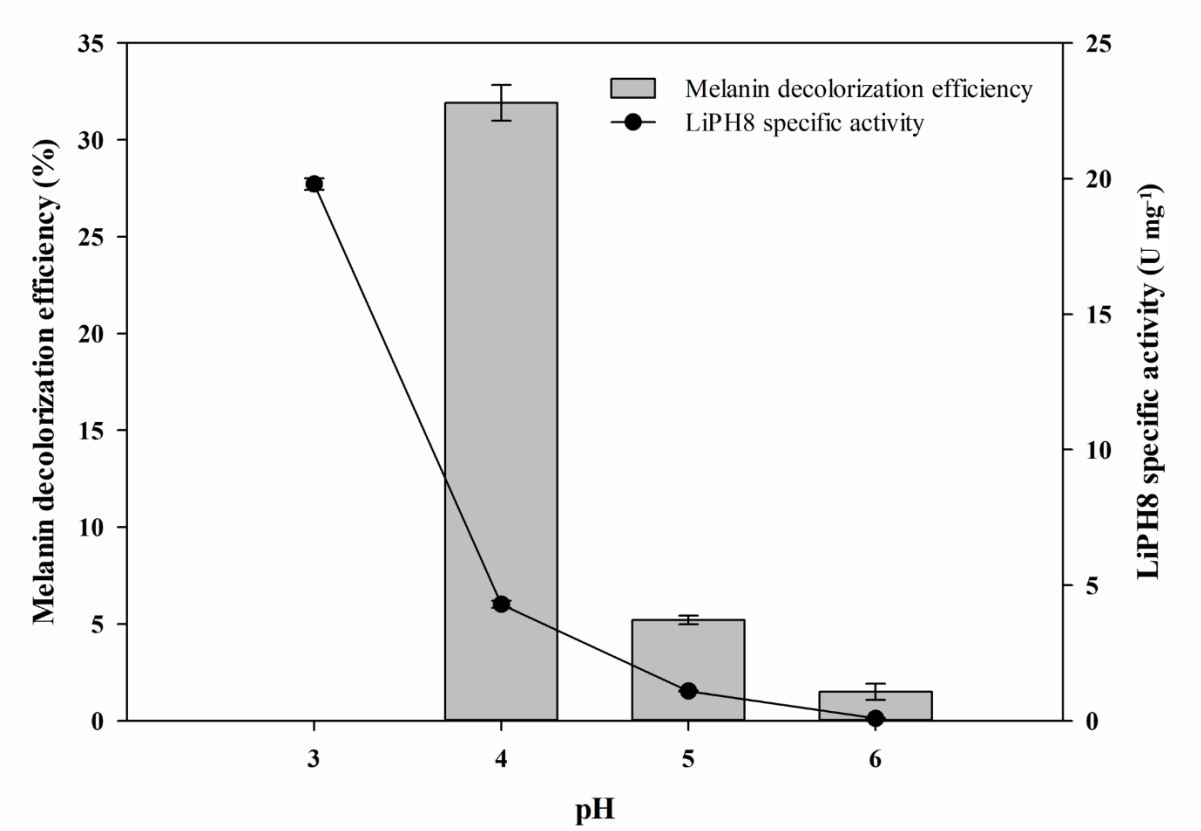


Figure 1. Effect of pH on melanin decolorization efficiency (bar graph, left axis) and specific activity of LiPH8 for VA (line graph, right axis). Melanin decolorization was conducted in BR buffer, 0.06 U/mL LiPH8, 250 μM H_2O_2, 50 mg/L synthetic melanin and 2 mM veratryl alcohol for 1 h. Specific activity of LiPH8 for veratryl alcohol was measured with 0.02 μM LiPH8, 250 μM H_2O_2 and 2 mM veratryl alcohol.

Table 1. Effect of ionic strength in BR buffer on melanin decolorization

Ionic strength of BR buffer (mM)	Melanin decolorization efficiency (%)	Picture of melanin decolorization
0	31.55 ± 0.60	
100	32.55 ± 0.40	
500	Melanin precipitation	
1000	Melanin precipitation	

Reaction was carried out under 0.06 U/mL LiPH8, 250 μM H_2O_2 2 mM veratryl alcohol, and 50 mg/L synthetic melanin in BR buffer pH 4.0 with different ionic strength.

2.2 Inhibition by excessive concentration of H_2O_2 on melanin decolorization

Since H_2O_2 acts as final electron acceptor of melanin decolorization by LiPH8, more H_2O_2 is needed for better melanin decolorization. However, lignin peroxidase is vulnerable to inactivation in high concentration of H_2O_2 by formation of compound III or heme rupturing [21]. It is reported that inactivation of lignin peroxidase by H2O2 can be subdued by VA [35] which is key mediator for decolorization of melanin (Scheme 1).

Melanin decolorization efficiency reached to 31.9 % with 250 μM of H_2O_2 and then decreased along with increasing concentration of 250 μM of H_2O_2 (Fig. 2). This result shows the inhibition effect of H_2O_2. However, the specific activity of LiPH8 for VA was increased as the H_2O_2 concentration increases which clearly suggests that H_2O_2 boosts the short-term reaction (within 1 min) and inhibits the long-term reaction like melanin decolorization (within 1 h). These results imply that additional H_2O_2 is required for higher melanin decolorization efficiency but should keep the concentration of H_2O_2 low. Therefore, the present study made an effort to supply low concentration of H_2O_2 continuously which may improve the overall melanin decolorization efficiency.

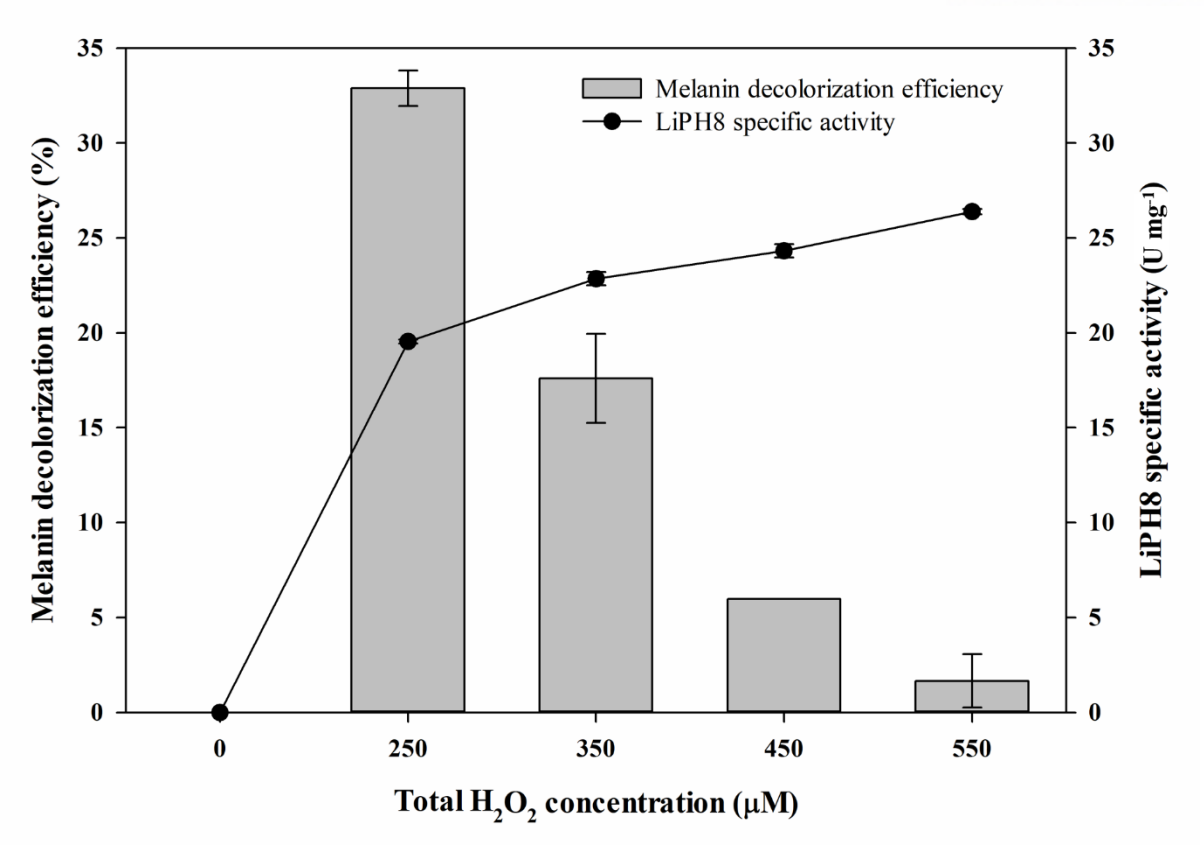


Figure 2. Effect of H_2O_2 concentrations melanin decolorization efficiency (bar graph, left axis) and specific activity of LiPH8 for VA (line graph, right axis). Melanin decolorization was conducted with 0.06 U/mL LiPH8, 50 mg/L synthetic melanin and 2 mM veratryl alcohol in BR buffer pH 4.0 for 1h. Specific activity of LiPH8 for veratryl alcohol was measured with 0.02 μM LiPH8 and 2 mM veratryl alcohol in pH 3.0 condition.

2.3 Effect of intermittently supplied H_2O_2 on melanin decolorization by LiPH8

The intermittent addition of H_2O_2 was carried out to support the assumption that the melanin decolorization efficiency can be improved by preserving H_2O_2 concentration low and keep supplying H_2O_2. As a result, melanin decolorization efficiency was reached to 73% which is more than twice as high as 31.9% in former experiment. The results showed that the critical point of H_2O_2 concentration which can inactivate LiPH8 is around 325 μM (Fig. 2, Fig. 3). However, melanin decolorization was inhibited in the case of 1400 μM of H_2O_2 with 12 min time interval even the instantaneous concentration of H_2O_2 was not exceeding 280 μM (Fig. 3). It seems that the amount of LiPH8 was not enough to catalyze H_2O_2 in 12 min. However, adding more enzyme will not improve melanin decolorization efficiency since it is saturated with around 1000 μM of H_2O_2 and approached to 73%. Except for the case, addition of H_2O_2 with lower instantaneous concentration than 300 μM did not hindered melanin decolorization by LiPH8. This result confirmed that melanin decolorization can be improved effectively by intermittent addition of H_2O_2 with time intervals which prevents inactivation of LiPH8. Still, it is not possible to spread H_2O_2 several times on the skin in case of cosmetic applications. Therefore, the present study suggested the *in-situ* generation of H_2O_2 to overcome this problem.

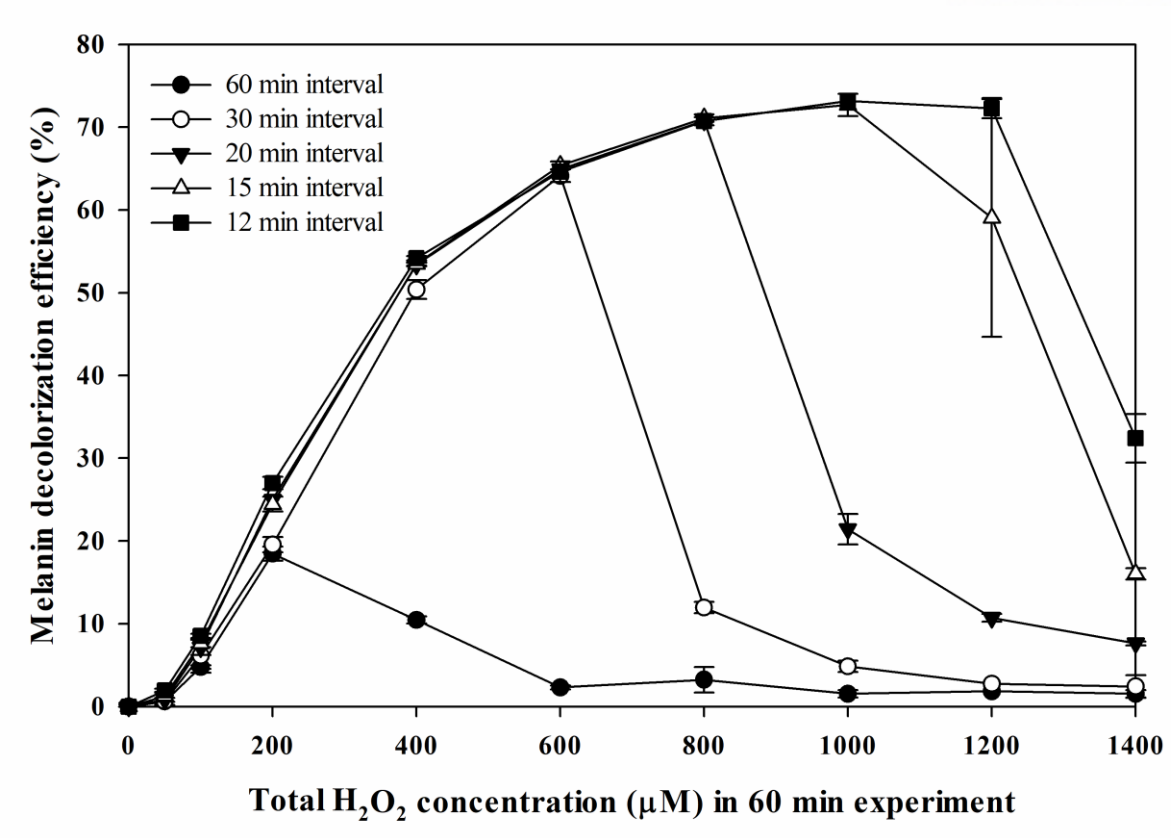


Figure 3. Decolorization of melanin with intermittently added H_2O_2. The reaction was conducted with 0.06 U/mL LiPH8, 50 mg/L synthetic melanin and 2 mM veratryl alcohol in pH 4.0 condition. The total H_2O_2 was divided equally into 1, 2, 3, 4 and 5 parts and added at every time intervals of 60 min (●), 30 min (○), 20 min (▼), 15 min (△) and 12 min (■) for 1 h.

2.4 LiPH8-catalyzed melanin decolorization using GOx for the generation of H_2O_2

Before the optimization, the control experiment was conducted by adding or not adding GOx, glucose, LiPH8, VA and H_2O_2. Meaningful melanin decolorization efficiency was detected only in two cases: reaction with LiPH8, VA and H_2O_2, reaction with GOx, glucose, LiPH8 and VA (Table 2). First case supports that melanin decolorization is done by LiPH8 and the second case supports that GOx and glucose can generate enough H_2O_2 for the reaction.

Glucose oxidase is known to produce H_2O_2 from glucose and molecular oxygen [36]. Thus, for the generation of H_2O_2, present study utilized glucose oxidase form *Aspergillus niger* (GOx). GOx is suitable for the melanin decolorization in pH 4.0 since it has optimum pH between pH 4.0 and pH 7.0 [37]. To analyze the influence of GOx concentration, melanin decolorization with LiPH8 was performed with varying units of GOx (Table 3) Melanin decolorization efficiency increased with the increase of GOx units. However, LiPH8 could not decolorize melanin without addition of GOx (Table 3). From this result, it is proven that GOx can generate H_2O_2 which is indispensable for melanin decolorization.

Effect of glucose concentration was also analyzed with the experiment in varying concentration of glucose since the generation of H_2O_2 is directly affected by it. As the concentration of glucose increases, melanin decolorization efficiency also increased and saturated around 100 mM of glucose (Table 4). However, minus value of decolorization efficiency was observed at the concentrations lower than 1 mM which implies that insufficient melanin oxidation resulted in intensifying of its color [38, 39]. The optimum concentration of GOx and glucose was obtained at the highest concentration of 0.24 U/mL and 300 mM respectively as shown in Table 3 and Table 4 since the substrates had no inhibition effects.

Melanin was gradually decolorized, and the color got lighter with time as shown in Fig. 4. Melanin decolorization efficiency reached 43.5% within 20 min and 63.3% in 1 h (Fig. 4). As the concentration of dark colored melanin decreases, the decolorization rate was also decreased. But LiPH8 was still active to achieve 84.0% even after 8 h and 86.9% at 24 h. Melanin decolorization efficiency could not achieve over 90% due to the deficiency of available melanin to be decolorized. These results strongly support that the GOx can supply low concentration and sufficient amount of H_2O_2 continuously which prevents LiPH8 from inactivation at least for 8 h. However, the human skin pigmentation spot model should be applied for future study since the genotoxicity or cytotoxicity of the substrates of melanin decolorization are not proven to be safe yet.

Table 2. The effect of reaction components of melanin decolorization

Reaction component added					Melanin decolorization efficiency (%)
GOx	**Glucose**	**LiPH8**	**VA**	**H_2O_2**	
+	–	–	–	–	-0.20 ± 0.00
–	+	–	–	–	-0.40 ± 0.28
–	–	+	–	–	-0.99 ± 0.00
–	–	–	+	–	-0.20 ± 0.00
+	+	–	–	–	0.59 ± 0.83
–	–	+	+	–	-2.24 ± 0.70
–	–	+	–	+	-1.01 ± 0.34
–	–	–	+	+	3.25 ± 1.40
–	–	+	+	+	32.89 ± 0.94
+	+	+	+	–	63.26 ± 2.36

0.24 U/mL GOx, 300 mM glucose, 0.06 U/mL of LiPH8, 2 mM veratryl alcohol and 250 μM H_2O_2 were used for the reactions in pH 4.0 condition.

Table 3. Decolorization of melanin with varying concentration of GOx

Glucose oxidase Unit (U/mL)	Melanin decolorization efficiency (%)
0	0
0.03	31.94 ± 0.42
0.06	49.70 ± 0.63
0.12	57.01 ± 2.96
0.24	63.43 ± 1.06

The reactions were carried out with 0.06 U/mL LiPH8, 300 mM glucose 50 mg/L synthetic melanin and 2 mM veratryl alcohol in pH 4.0 condition.

Table 4. Decolorization of melanin with varying concentration of glucose

Glucose concentration (mM)	Melanin decolorization efficiency (%)
0	0
0.1	-2.35 ± 0.44
0.5	-0.78 ± 0.44
1	-0.78 ± 0.44
5	21.35 ± 1.11
10	47.10 ± 0.67
20	57.30 ± 0.44
100	62.48 ± 0.22
300	62.17 ± 4.22

The reactions were carried out with 0.06 U/mL LiPH8, 0.24 GOx, 2 mM veratryl alcohol and 50 mg/L synthetic melanin in pH 4.0 condition.

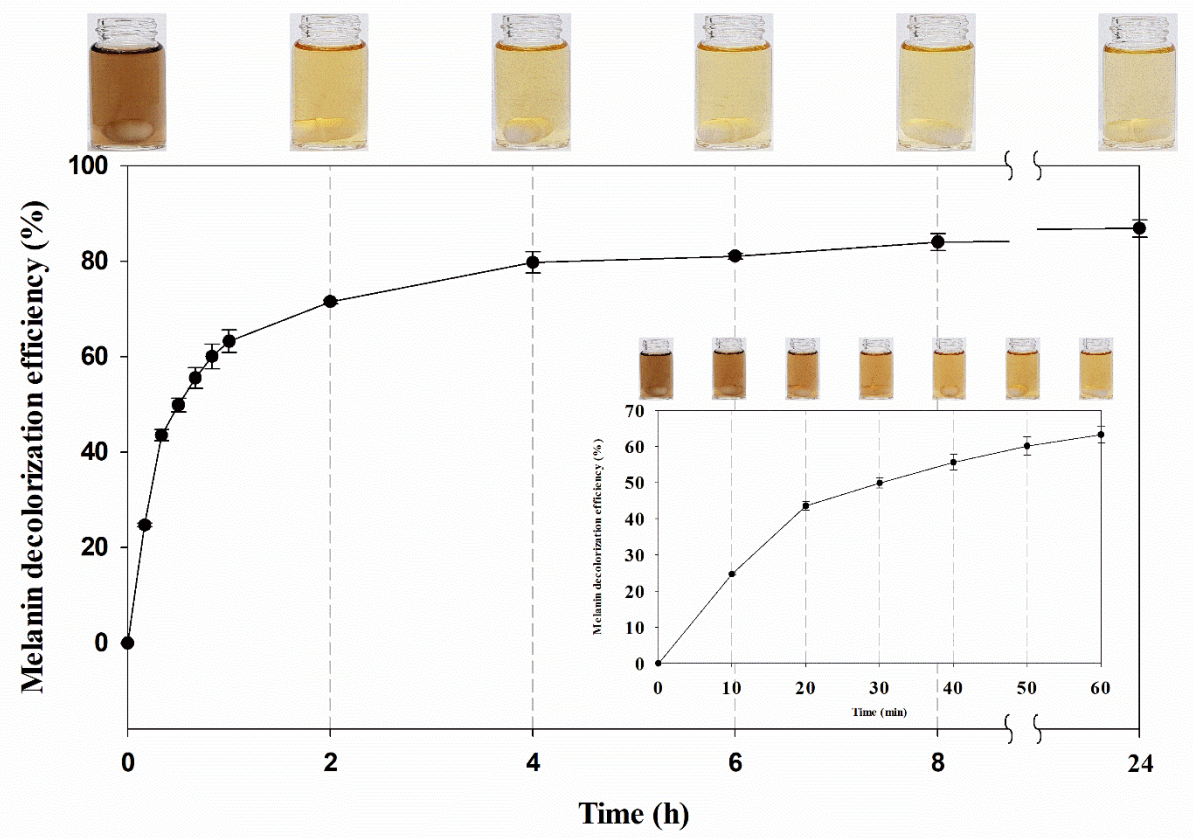


Figure 4. The time profile of melanin decolorization efficiency catalyzed by LiPH8 and GOx. The melanin decolorization time profile in 1 h is included as inset. Melanin decolorization was conducted in pH 4.0 condition, 0.06 U/mL LiPH8, 300 mM glucose, 0.24 U/mL glucose oxidase, 50 mg/L synthetic melanin and 2 mM veratryl alcohol. The pictures on the top of graphs are the reaction solution after decolorization.

3. Conclusion

The present work aims to show the effectiveness and efficiency of recombinant LiPH8 expressed in *E. coli* for melanin decolorization. LiPH8 and GOx were co-catalyzed to improve the melanin decolorization efficiency. LiPH8 was able to get over the inhibition effect of excessive H_2O_2 concentration with GOx by supplying low concentration of H_2O_2 continuously. Through this method, melanin decolorization efficiency reached 63.3 % within 1 h and achieved 84.0 % in 8 h which proved the potential of co-catalysis of LiPH8 and GOx in whitening cosmetic formulation.

4. Materials and methods

4.1 Materials

Hydrogen peroxide was purchased from Daejung Chemicals & Metals, South Korea. β-D-glucose, boric acid (H_3BO_3), calcium chloride ($CaCl_2$), glucose oxidase from Aspergillus niger (GOx), hemin, L-glutathione oxidized, potassium chloride (KCl), sodium hydroxide (NaOH), synthetic melanin, trizma base, trizma hydrochloride and urea were obtained from Sigma-aldrich, USA. Expression vector pET-21b (+) (Novogene, USA) and *Escherichia coli* BL21 (DE3) (Real Biotech Corporation, Taiwan) were employed for the overexpression of protein. BugBuster® Master Mix (71456-4) was procured for cell lysis from EMD Millipore, USA. For the induction of gene expression, isopropyl β-D-1-thiogalactopyranoside (IPTG) was obtained from Duchefa Biochemie, Netherlands. HiTrap Q HP anion exchange column was utilized for fast protein liquid chromatography (FPLC), which was purchased from GE Healthcare BioSciences, USA. Guanidine chloride, phosphoric acid (H_3PO_4), sodium acetate (CH_3COONa) and veratryl alcohol (3,4-dimethoxybenzyl alcohol or VA) were obtained from Tokyo Chemical Industry (TCI), Japan. Acetic acid (CH_3COOH) was procured from Junsei, Japan.

4.2 Expression and purification of recombinant lignin peroxidase H8

The gene encoding LiPH8 from *Phanerochaete chrysosporium* (UniprotKB entry: P06181) was optimized and synthesized by Bioneer (South Korea). Expression, refolding and purification procedures were derived from previous research [40]. The synthetic gene encoding LiPH8 was subcloned in pET-21b (+) expression vector and transformed to *E. coli* BL21 (DE3) for heterologous expression of LiPH8 [41]. 1mM IPTG was added for induction and cell was harvested. BugBuster® Master Mix was used for cel lysis. Inclusion body from lysate was obtained by centrifuge (11000 rpm, 4 °C, 10 min). Sonication (Pulse on and off: 1 s and 2 s, amplitude: 20% for 2 min) and additional centrifugation (11000 rpm, 4 °C, 10 min) was conducted for three times to remove impurities and to get clean inclusion bodies. The color of inclusion bodies should be monitored to prevent burnt of it. 8 M urea was used to dissolve the inclusion bodies. The refolding was carried out in the condition of 0.1 Tris-buffer (pH 8.5), 25 μM hemin, 0.71 mM L-glutathione oxidized form, 1.8 mM $CaCl_2$ and 0.36 M guanidine hydrochloride. The refolded protein was dialyzed and purified through ÄKTA FPLC chromatography system (GE Healthcare Life Sciences, USA) with HiTrap Q HP column. After purification, LiPH8 was dialyzed and stored in pH 6.0, 10 mM sodium acetate buffer.

4.3 Lignin peroxidase activity assay for VA

The enzyme activity assay was conducted in Britton-Robinson buffer (BR buffer) with ionic strength of 0.1 M and pH 3.0 as initial experiment to set the standard [42], 0.02 μM LiPH8, 2 mM VA and 250 μM H_2O_2 using spectrophotometer (UV-1650PC, Shimadzu, Japan) at 310 nm within 1 min. The temperature was fixed to 25°C by using circulator. Specific activity was calculated with extinction coefficient of veratraldehyde ($\varepsilon_{310\ nm}$ = 9.3 mM^{-1} cm^{-1}) since one veratryl alcohol converts to one veratraldehyde [43]. For specific activity assay with different pH, above-mentioned concentration of LiPH8, H_2O_2 and VA were used in 0.1 M BR buffer with varying pH from 3.0 to 6.0. Effect of H_2O_2 concentrations ranging 0 μM -550 μM was also analyzed. One unit LiPH8 is the amount of enzyme which can oxidize 1 μmol of VA and turn into veratraldehyde per minute.

4.4 Melanin decolorization catalyzed by LiPH8

Melanin decolorization with varying pH was carried out in BR buffer ranging from pH 3.0 to pH 6.0 with 0.06 U/mL LiPH8 and 250 μM H_2O_2, 2 mM VA for 1 h. All the melanin decolorization experiments are conducted with 50 mg/L synthetic melanin under 100 rpm stirring with magnetic bar at room temperature with no exception. The ionic strength was controlled with KCl concentration and the effect of ionic strength ranging from 0 mM to 1000 mM on melanin decolorization was also investigated. The reaction was begun with the addition of H_2O_2 in reaction mixture contains BR buffer, LiPH8, VA and synthetic melanin with 2 mL of final volume. The unit of LiPH8 was determined by initially measured enzyme activity for VA at pH 3.0.

The melanin decolorization efficiency was estimated from the absorbance difference between reaction solution and control solution. Absorbance was measured by UV/vis spectrophotometer at 540 nm wavelength after 1 h reaction. Melanin decolorization efficiency was calculated using Eq. (1). Only H_2O_2 was excluded for the control experiment compare to the reaction solution. All the experiments were conducted twice and reported as mean ± S.D.

$$\text{Decolorization efficiency (\%)} = \frac{A_{540\ control} - A_{540\ after\ 1h\ reaction}}{A_{540\ control}} \times 100 \quad (1)$$

Melanin decolorization to analyze the inhibition effect of excessive H_2O_2 concentration was conducted using 0.06 U/mL of LiPH8, 2 mM VA and 50 mg/L synthetic melanin in pH 4.0 BR buffer with varying concentrations of H_2O_2 from 0 μM to 550 μM for 1 h.

4.5 Melanin decolorization catalyzed by LiPH8 with intermittent addition of H_2O_2

The melanin decolorization was carried out in pH 4.0 BR buffer with aforementioned condition except for H_2O_2. Total concentration of H_2O_2 up to 1400 μM was divided and added with 12 min, 15 min, 20 min, 30 min and 60 min intervals for 1 hour to support that the instantaneously high concentration of H_2O_2 is the major inhibition factor of LiPH8.

4.6 Melanin decolorization catalyzed by LiPH8 with in-situ generated H_2O_2 by GOx

GOx was applied for H_2O_2 generation on decolorization of melanin by LiPH8 instead of adding H_2O_2 directly. Decolorization of melanin was performed in BR buffer (pH 4.0), 300 mM β-D-glucose with glucose oxidase ranging from 0 U/mL to 0.24 U/mL rather than adding H_2O_2. LiPH8, VA and synthetic melanin concentration was fixed as previous section. BR buffer was vortexed to dissolve enough molecular oxygen for GOx. The reaction was initiated with the addition of GOx in 2 mL reaction mixture. Definition of one unit GOx is the amount of enzyme which catalyzes glucose and produce 1 μmol of H_2O_2 per minute. The GOx unit was determined based on the label of product. Glucose concentrations from 0 mM to 300 mM were used to analyze the effect of glucose varying concentrations on decolorization of melanin with aforementioned condition for 1 h. The control experiment was conducted with identical condition as mentioned above without adding glucose oxidase. The control experiments for all the other substances were also conducted to figure out the effect of each and every component of the reaction such as GOx, glucose, LiPH8, VA and H_2O_2. All the reactions were performed twice and reported as mean ± S.D.

4.7 Time profile of melanin decolorization catalyzed by LiPH8 using in-situ generated H_2O_2

The reaction was carried out with 0.06 U/mL LiPH8, 0.24 U/mL GOx, 300 mM glucose, 50 mg/L synthetic melanin and 2 mM VA in pH 4.0 BR buffer for 24 h for the melanin decolorization time profile. The reaction was started with the addition of glucose oxidase. The reaction solution was collected every 10 min for the first 1 h than at 2 h, 4 h, 6 h, 8 h and 24 h. The decolorization efficiency was measured immediately with spectrophotometer at 540 nm.

References

1. Sturm, R. A., Teasdale, R. D., & Box, N. F. (2001). Human pigmentation genes: identification, structure and consequences of polymorphic variation. Gene, 277(1-2), 49-62.
2. Kaidbey, K. H., Agin, P. P., Sayre, R. M., & Kligman, A. M. (1979). Photoprotection by melanin—a comparison of black and Caucasian skin. Journal of the American Academy of Dermatology, 1(3), 249-260.
3. Stępień, K., Dzierżęga-Lęcznar, A., Kurkiewicz, S., & Tam, I. (2009). Melanin from epidermal human melanocytes: study by pyrolytic GC/MS. Journal of the American Society for Mass Spectrometry, 20(3), 464-468.
4. Hennessy, A., Oh, C., Diffey, B., Wakamatsu, K., Ito, S., & Rees, J. (2005). Eumelanin and pheomelanin concentrations in human epidermis before and after UVB irradiation. Pigment cell research, 18(3), 220-223.
5. Smit, N., Vicanova, J., & Pavel, S. (2009). The hunt for natural skin whitening agents. International journal of molecular sciences, 10(12), 5326-5349.
6. Zaidi, K. U., Ali, A. S., Ali, S. A., & Naaz, I. (2014). Microbial tyrosinases: promising enzymes for pharmaceutical, food bioprocessing, and environmental industry. Biochemistry research international, 2014.
7. Chang, T. S. (2009). An updated review of tyrosinase inhibitors. International journal of molecular sciences, 10(6), 2440-2475.
8. Lim, J. T. E. (1999). Treatment of melasma using kojic acid in a gel containing hydroquinone and glycolic acid. Dermatologic surgery, 25(4), 282-284.
9. Nazzaro-Porro, M., & Passi, S. (1978). Identification of tyrosinase inhibitors in cultures of Pityrosporum. *Journal of Investigative Dermatology*, *71*(3), 205-208.
10. Jin, Y. H., Lee, S. J., Chung, M. H., Park, J. H., Park, Y. I., Cho, T. H., & Lee, S. K. (1999). Aloesin and arbutin inhibit tyrosinase activity in a synergistic manner via a different action mechanism. Archives of pharmacal research, 22(3), 232.
11. Lee, M. H., Lin, Y. P., Hsu, F. L., Zhan, G. R., & Yen, K. Y. (2006). Bioactive constituents of Spatholobus suberectus in regulating tyrosinase-related proteins and mRNA in HEMn cells. Phytochemistry, 67(12), 1262-1270.

12. Tanemura, A., Yang, L., Yang, F., Nagata, Y., Wataya-Kaneda, M., Fukai, K., ... & Katayama, I. (2015). An immune pathological and ultrastructural skin analysis for rhododenol-induced leukoderma patients. Journal of dermatological science, 77(3), 185-188.

13. Tian, Y., Hoshino, T., Chen, C. J., E, Y., Yabe, S., & Liu, W. (2009). The evaluation of whitening efficacy of cosmetic products using a human skin pigmentation spot model. Skin Research and Technology, 15(2), 218-223.

14. Khammuang, S., & Sarnthima, R. (2013). Decolorization of synthetic melanins by crude laccases of Lentinus polychrous Lév. Folia microbiologica, 58(1), 1-7.

15. Falade, A. O., Mabinya, L. V., Okoh, A. I., & Nwodo, U. U. (2019). Biochemical and molecular characterization of a novel dye-decolourizing peroxidase from Raoultella ornithinolytica OKOH-1. International journal of biological macromolecules, 121, 454-462.

16. Nagasaki, K., Kumazawa, M., Murakami, S., Takenaka, S., Koike, K., & Aoki, K. (2008). Purification, characterization, and gene cloning of Ceriporiopsis sp. strain MD-1 peroxidases that decolorize human hair melanin. Appl. Environ. Microbiol., 74(16), 5106-5112.

17. Mohorčič, M., Friedrich, J., Renimel, I., André, P., Mandin, D., & Chaumont, J. P. (2007). Production of melanin bleaching enzyme of fungal origin and its application in cosmetics. Biotechnology and Bioprocess Engineering, 12(3), 200-206.

18. Woo, S. H., Cho, J. S., Lee, B. S., & Kim, E. K. (2004). Decolorization of melanin by lignin peroxidase from Phanerochaete chrysosporium. Biotechnology and bioprocess engineering, 9(4), 256.

19. Eom, M. H., & Kim, Y. H. (2014). Inactivating effect of phenolic unit structures on the biodegradation of lignin by lignin peroxidase from Phanerochaete chrysosporium. Enzyme and microbial technology, 61, 48-54.

20. Passardi, F., Theiler, G., Zamocky, M., Cosio, C., Rouhier, N., Teixera, F., ... & Dunand, C. (2007). PeroxiBase: the peroxidase database. Phytochemistry, 68(12), 1605-1611.

21. Chung, N., & Aust, S. D. (1995). Inactivation of lignin peroxidase by hydrogen peroxide during the oxidation of phenols. Archives of Biochemistry and Biophysics, 316(2), 851-855.

22. Pillaiyar, T., Manickam, M., & Namasivayam, V. (2017). Skin whitening agents: Medicinal chemistry perspective of tyrosinase inhibitors. Journal of enzyme inhibition and medicinal chemistry, 32(1), 403-425.

23. Watanabe, F., Hashizume, E., Chan, G. P., & Kamimura, A. (2014). Skin-whitening and skin-condition-improving effects of topical oxidized glutathione: a double-blind and placebo-controlled clinical trial in healthy women. Clinical, cosmetic and investigational dermatology, 7, 267.

24. Mauricio, T., Karmon, Y., & Khaiat, A. (2011). A randomized and placebo-controlled study to compare the skin-lightening efficacy and safety of lignin peroxidase cream vs. 2% hydroquinone cream. Journal of cosmetic dermatology, 10(4), 253-259.

25. Hu, Z. M., Zhou, Q., Lei, T. C., Ding, S. F., & Xu, S. Z. (2009). Effects of hydroquinone and its glucoside derivatives on melanogenesis and antioxidation: Biosafety as skin whitening agents. Journal of dermatological science, 55(3), 179-184.

26. Menter, A. (2004). Rationale for the use of topical corticosteroids in melasma. Journal of drugs in dermatology: JDD, 3(2), 169-174.

27. Ortonne, J. P. (2006). Retinoid therapy of pigmentary disorders. Dermatologic therapy, 19(5), 280-288.

28. Jergil, B., Lindbladh, C., Rorsman, H., & Rosengren, E. (1984). Inactivation of human tyrosinase by cysteine. Protection by dopa and tyrosine. Acta dermato-venereologica, 64(2), 155-157.

29. Burger, P., Landreau, A., Azoulay, S., Michel, T., & Fernandez, X. (2016). Skin whitening cosmetics: Feedback and challenges in the development of natural skin lighteners. Cosmetics, 3(4), 36.

30. Rafiee, M., & Javaheri, M. (2015). A theoretical study of benzaldehyde derivatives as tyrosinase inhibitors using Ab initio calculated NQCC parameters. Molecular Biology Research Communications, 4(3), 151.

31. Adrio, J. L., & Demain, A. L. (2014). Microbial enzymes: tools for biotechnological processes. Biomolecules, 4(1), 117-139.

32. Shimada, M., Nakatsubo, F., Kirk, T. K., & Higuchi, T. (1981). Biosynthesis of the secondary metabolite veratryl alcohol in relation to lignin degradation in Phanerochaete chrysosporium. Archives of Microbiology, 129(4), 321-324.

33. Khindaria, A., Yamazaki, I., & Aust, S. D. (1996). Stabilization of the veratryl alcohol cation radical by lignin peroxidase. Biochemistry, 35(20), 6418-6424.

34. Candeias, L. P., & Harvey, P. J. (1995). Lifetime and reactivity of the veratryl alcohol radical cation. Implications for lignin peroxidase catalysis. Journal of Biological Chemistry, 270(28), 16745-16748.

35. Cancel, A. M., Orth, A. B., & Tien, M. (1993). Lignin and veratryl alcohol are not inducers of the ligninolytic system of Phanerochaete chrysosporium. Appl. Environ. Microbiol., 59(9), 2909-2913.

36. Leskovac, V., Trivić, S., Wohlfahrt, G., Kandrač, J., & Peričin, D. (2005). Glucose oxidase from Aspergillus niger: the mechanism of action with molecular oxygen, quinones, and one-electron acceptors. The international journal of biochemistry & cell biology, 37(4), 731-750.

37. Kalisz, H. M., Hecht, H. J., Schomburg, D., & Schmid, R. D. (1991). Effects of carbohydrate depletion on the structure, stability and activity of glucose oxidase from Aspergillus niger. Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology, 1080(2), 138-142.

38. Plonka, P. M., & Grabacka, M. (2006). Melanin synthesis in microorganisms-biotechnological and medical aspects. Acta Biochimica Polonica, 53(3), 429-443.

39. Rogic, M. M., & Demmin, T. R. (1978). Cleavage of carbon-carbon bonds. Copper (II)-induced oxygenolysis of o-benzoquinones, catechols, and phenols. On the question of nonenzymic oxidation of aromatics and activation of molecular oxygen. Journal of the American Chemical Society, 100(17), 5472-5487.

40. Doyle, W. A., & Smith, A. T. (1996). Expression of lignin peroxidase H8 in Escherichia coli: folding and activation of the recombinant enzyme with Ca2+ and haem. Biochemical journal, 315(1), 15-19.

41. Seo, H., Kim, K. J., & Kim, Y. H. (2018). In silico-designed lignin peroxidase from Phanerochaete chrysosporium shows enhanced acid stability for depolymerization of lignin. Biotechnology for Biofuels, 11(1), 325.

42. Mongay, C. A. R. L. O. S., & Cerda, V. (1974). Britton–Robinson buffer of known ionic strength. Anal Chim, 64, 409-412.

43. Tien, M., & Kirk, T. K. (1984). Lignin-degrading enzyme from Phanerochaete chrysosporium: purification, characterization, and catalytic properties of a unique H2O2-requiring oxygenase. Proceedings of the National Academy of Sciences, 81(8), 2280-2284

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